**REMARKS**

In the foregoing Listing of Claims, Applicants cancel claim 1 and amend claims 18 and 22 by further defining a method of inhibiting tyrosinase activity in a subject in need of preventing or alleviating spots and freckles created on skin or a method of ameliorating facial blood flow in a subject in need thereof, which comprises administering a composition comprising an effective amount of anthocyan for suppressing preventing or alleviating spots and freckles created on skin or for ameliorating facial blood flow to the subject. These aspects of Applicants' invention are described on page 5, lines 6-18; page 8, lines 12-17; and elsewhere in the Specification. Applicants respectfully request reconsideration and allowance of the inventions defined in claims 18-25 for reasons that follow.

Applicants desire to express thanks to Examiner Elli Peselev for the courtesies extended the undersigned in a telephone interview on January 22, 2010. During the interview, the foregoing amendments to claims 18 and 22 were discussed among other things including the alleged inherency of the presently claimed method within the teachings of Matsumoto (EP 1 208 755 A1). Examiner Peselev stated that amended claim 22 has a very good chance of patentability. With respect to amended claim 18, Examiner Peselev stated this claim has a good chance of patentability, but she would have to consider this matter further after a response is filed.

The Office Action included a single prior art rejection of claims 1 and 18-25 under 35 U.S.C. §102(b) as being anticipated by Matsumoto. Matsumoto was used to reject Applicants' claims in previous Office Actions. The Office Action took the position that Applicants' claimed tyrosinase inhibiting activity and amelioration of facial blood flow activity would have been inherent in the method disclosed by Matsumoto. In the foregoing amendments, Applicants

cancel claim 1. In addition, Applicants amend claim 18 by defining a method of inhibiting tyrosinase activity in a subject in need of preventing or alleviating spots and freckles created on skin, which comprises administering a composition comprising an effective amount of anthocyan for preventing or alleviating spots and freckles created on skin to the subject. Similarly, Applicants amend claim 22 by defining a method of ameliorating facial blood flow in a subject in need thereof, which comprises administering a composition comprising an effective amount of anthocyan for ameliorating facial blood flow to the subject. Applicants respectfully submit that the presently claimed method of inhibiting tyrosinase activity in a subject in need of preventing or alleviating spots and freckles created on skin and the presently claimed method of ameliorating facial blood flow in a subject in need thereof, which comprises administering a composition comprising an effective amount of anthocyan for preventing or alleviating spots and freckles created on skin or for ameliorating facial blood flow to the subject, are not and cannot be inherent within the teachings of Matsumoto. Therefore, the presently claimed methods as defined in claims 18-25 cannot be anticipated by Matsumoto within the meaning of 35 U.S.C. §102.

Applicants respectfully submit that the presently claimed use of inhibiting tyrosinase activity in a subject in need of preventing or alleviating spots and freckles created on skin as required in present claims 18-21 is not related to the uses of improving visual function, improving body fluidity, and/or lowering blood pressure as discussed in Matsumoto, and thus is a new use over Matsumoto.

Applicants speculate that the mechanism of inhibiting tyrosinase is as follows. When epidemic cells are irradiated with ultraviolet light, a signaling substance that enhances the synthesis of melanine is produced. The signaling substance binds to melanocytes. The

melanocytes are then activated and grow, and produce and activate tyrosinase that is a melanine-producing enzyme. The activated tyrosinase converts tyrosine to DOPA (dihydroxyphenylalanine) in a living body and then dopaquinone which stimulates the production of melanine is produced. Accordingly, inhibiting tyrosinase inhibits the production of DOPA and dopaquinone, which in turn inhibit the production of melanine. The attached Exhibit A (K. Ohhara et al., Functional Food, 2009, Vol. 2, No. 4, p. 383-386) shows the mechanism, especially in Fig. 3.

The teachings of Matsumoto never disclose nor suggest the tyrosinase-inhibiting activity of anthocyanin and any relationship between tyrosinase-inhibiting activity and the production of melanine in a subject in need of preventing or alleviating spots and freckles created on skin as required in present claims 18 to 21. In addition, these properties or functions of the inventions defined in claims 18 to 21 are not related to the uses of improving visual function, improving body fluidity, and/or lowering blood pressure as proposed by Matsumoto, and thus is a new use or utility over Matsumoto. At least for these reasons, Applicants respectfully submit that the inventions of claims 18 to 21 are not anticipated by Matsumoto, and thus, are patentable thereover.

Applicants' claims 22 to 25 are directed to a method of ameliorating facial blood flow in a subject in need thereof, which comprises administering a composition comprising an effective amount of anthocyan for ameliorating facial blood flow to the subject. This claimed use is not related to the uses of improving visual function, improving body fluidity, and/or lowering blood pressure as discussed in Matsumoto, and thus is a new use over Matsumoto.

At best, Matsumoto proposes that an anthocyanin-containing composition has a blood fluidity improvement function. However, the blood fluidity improvement function discussed in

Matsumoto is different from the function for improving facial blood flow that is required in the present claims. In particular, Matsumoto describes the blood fluidity effects in Example 12. Example 12 discloses "This fresh whole blood obtained by the collection of heparin was poured into a micro channel array (width 7 μm, height 30 μm, depth 4.5 μm, and 8736 channels (Bloody 6-7, Hitachi Haramachi Electronics Co., Ltd.) at a water column difference of 20cm using MC-FAN (Santuri Kiko). The time necessary for 100 μl to pass through was determined." That is, the "improving blood fluidity function" is evaluated by collecting blood measuring the time for blood to pass through the micro channel array. Attached Exhibit B is a copy of the catalogue of the MC-FAN used in the experiment of Example 12. Pages 2 and 4 of Exhibit B show the micro channel array. The width of the micro channel array is 7 μm, which is the same as the diameter of a blood capillary. Page 2 of Exhibit B includes examples that describe how the analyzer is used. It is clear that the fluidity of blood component such as erythrocytes, leukocytes, and platelets was measured in Example 12 of Matsumoto. That is, the alleged "blood fluidity improvement function" of Matsumoto is a function to improve the fluidity of blood components such as erythrocytes, leukocytes, and platelets. Furthermore, Matsumoto describes, "That is, according to the present invention, diseases such as cerebral ..... by affecting erythrocytes, leukocytes, and platelets as such in the blood to improve the fluidity of the blood itself, thereby lowering blood pressure rather than by vasoconstriction" in paragraph [0095] of EP 1208755 A1.

On the contrary, the blood flow improving function of the presently claimed inventions is based on vasodilatation effect on peripheral vessels. This function is significantly different from and unrelated to the blood fluidity improvement function discussed in Matsumoto.

The attached Exhibit C (Iwasaki-Kurashige et al., Vascular Pharmacology 44 (2006) 215-223) shows that blackcurrant concentrate which includes anthocyanin decreases peripheral vascular resistance that results in vasodilatation. Exhibit C illustrates the blood flow improving function required in claims 22-25. Furthermore, the presently claimed method has the advantageous effect of ameliorating facial blood flow within 15 minutes.

Accordingly, the claimed inventions in claims 18 to 21 are based on the newly found function of anthocyanin for improving facial blood flow, which function is not describe nor inherent in Matsumoto. It is well established in the case law that the discovery of a new use for an old structure based on unknown properties of the structure might be patentable to the discoverer as a process of using. *In re Hack*, 245 F.2d 246, 248, 114 USPQ 161, 163 (CCPA 1957). In US patent practice, many patents have been issued for novel use of known substances. For example, minoxydil had been patented as a blood pressure-towering drug (US 3,461,461). Then, the new effect of minoxydil for enhancing hair growth was discovered and minoxydil was patented as a hair growth stimulant (US 4,139,619). Applicants respectfully submit that the methods defined in claims 18-25 fall within this category of invention. Namely, the presently claimed method of inhibiting tyrosinase activity in a subject in need of preventing or alleviating spots and freckles created on skin and the presently claimed method of ameliorating facial blood flow in a subject in need thereof, which comprise administering a composition comprising an effective amount of anthocyan for preventing or alleviating spots and freckles created on skin or for ameliorating facial blood flow to the subject, are new uses for the compounds set forth in the present claims, which are not and cannot be inherent within the teachings of Matsumoto. At least for this reason, the inventions defined in claims 18-25, which are based on new uses of anthocyanin, are patentable.

At least for the foregoing reasons, Applicants respectfully submit that the presently claimed invention is patently distinguishable from Matsumoto. Therefore, Applicants respectfully request that the Examiner reconsider and withdraw any §102 or §103 rejection of method claims 18-25 over the teachings of Matsumoto.

Applicants believe that the foregoing is a complete and proper response to the Office Action mailed September 23, 2009. While it is believed that all pending claims in this application are in condition for allowance, if the Examiner has any comments or questions, Applicants invite the Examiner to telephone the undersigned to resolve any outstanding issues at the below listed number.

In the event this paper is not timely filed, Applicants hereby petition for an appropriate extension of time. The Commissioner is hereby authorized to charge the fee therefor, as well as any other fees which become due, to our Deposit Account No. 50-1147.

Respectfully submitted,

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ATTACHMENTS:

EXHIBIT A ~ K. Ohhara et al., Functional Food, 2009, Vol. 2, No. 4, p. 383-386 (5 pp.).
EXHIBIT B ~ Catalogue of MC-FAN (4 pp).
EXHIBIT C ~ Iwasaki-Kurashige et al., Vascular Pharmacology 44 (2006) 215-2236 (6 pp.).

Exhibit A
Partial English translation of K. Ohhara et al., Functional Food, 2009, Vol.2, No.4, p.383-386

**Partial translation of K. Ohhara et al., Functional Food, 2009, Vol.2, No.4, p.383-386**

Special topic Aging of skin and functional food

6. Effects of functional components in food for improving and preventing spots and cockles

Hiroki Ohhara[1], Shingo TAJIMA[2]

1) Meiji Seika Kaisha, Ltd, Food and Healthcare Research Institute
2) National Defense Medical College Hospital

Abstract
lines 6 to 8

It has been suggested that cassis-anthocyanin components which are transferred in blood inhibits tyrosinase activity which involves in producing melanine and ameliorating impaired blood circulation and takes effect on spots.

Figure 3 Hypothesis for effects of cassis-anthocyanin on improving and preventing spots

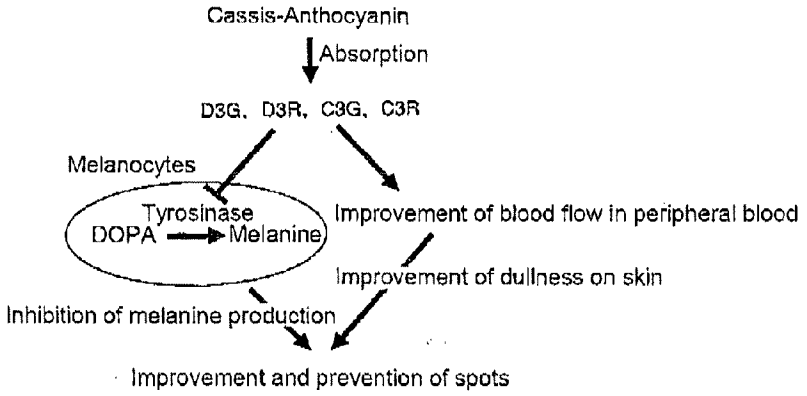



Exhibit A
page 1
(English

Exhibit A

特集 皮膚老化と機能性食品

# 6. 食品機能成分のシミ，シワの改善と予防効果

大原 浩樹[1)]　多鹿 新吾[2)]

[1)] 明治製菓株式会社食料健康総合研究所
[2)] 防衛医科大学校皮膚科学講座

皮膚の老化に伴って発生するシミやシワを改善・予防することはQOL（quality of life）向上にも重要である。近年，予防的な観点から食品機能成分の皮膚に対する効果について研究が進められ，その生理機能が明らかになってきている。そこで，我々はカシスアントシアニンとコラーゲンペプチドについて研究を進め，シミとシワの改善・予防効果を見出した。

カシスアントシアニンは，血中に移行したカシスアントシアニン成分がメラニン産生に関与するチロシナーゼ活性を阻害し，さらに血流不全を改善することでシミに対して効果があることが示唆された。一方，コラーゲンペプチドは，血中に移行したHyp（ヒドロキシプロリン）含有ペプチドが線維芽細胞の細胞外マトリクス産生に関与し，さらに表皮の水分低下，バリア機能低下を改善することでシワに対して効果があることが示唆された。

●キーワード　食品機能成分，シミ，シワ，カシスアントシアニン，コラーゲンペプチド

## はじめに

現在，高齢化社会のさらなる伸進が推測され，皮膚科領域においても老化・加齢変化に関する研究が進められている。この皮膚の老化は，内因性老化と外因性老化に分けられる。内因性老化とは生理的老化ともいい，各個人の遺伝子的素因を背景に生じる皮膚の加齢に伴う老化であり，形態的変化，機能的老化として表れる。一方，外因性老化とは内因性老化に環境因子，喫煙，紫外線照射による光老化などの環境要因による皮膚障害のダメージが蓄積して生じる

1882-3971/09/¥500/和文/JCLS



Exhibit A
page 2

老化である。外因性老化の光老化に関しては，日光に当たることの多い顔面などの露光部で顕著であり，シミ，シワなどの徴候として表れる。この皮膚の老化に伴って発生するシミやシワを改善・予防することはQOL（quality of life）向上の観点からも重要であり，近年，食品機能成分を用いた改善・予防効果の研究が精力的に進められている。

## 1 シミの改善と予防効果

シミは皮膚基底層に存在するメラノサイトから産生される高分子色素メラニンが沈着し，発生したものである。メラニン産生の原因の一つとしては，紫外線照射が挙げられる。このメラニンは，メラノサイト内のメラノソームにおいてチロシナーゼが作用することで，チロシン，ドーパ（Dopa），ドーパクロム（Dopachrome）を経て合成される[1]。このチロシナーゼ活性を阻害することができればメラニン合成を抑制することができ，シミの改善・予防が可能となる。また，上述のメラニンの産生以外にも，顔面の血流不全によるくすみもシミの原因であると考えられている[2]。

このシミを改善・予防する食品機能成分としては，ビタミンC，L-システイン，コウジ酸などの効果が知られている。コウジ酸に関しては，肝臓への影響の問題から2003年以降使用が中止されたものの，ビタミンC，L-システインを配合した食品やサプリメントは多く市販されている。最近，我々はカシスアントシアニンのシミに対する予防・改善を示唆する結果を得たので，以下，カシスアントシアニンのシミに対する作用について概説する。

カシスは欧州で消費量の多いベリー果実であり，豊富に含まれるポリフェノールの一種であるD3G（アントシアニンはデルフィニジン-3-グルコシド），D3R（デルフィニジン-3-ルチノシド），C3G（シアニジン-3-グ

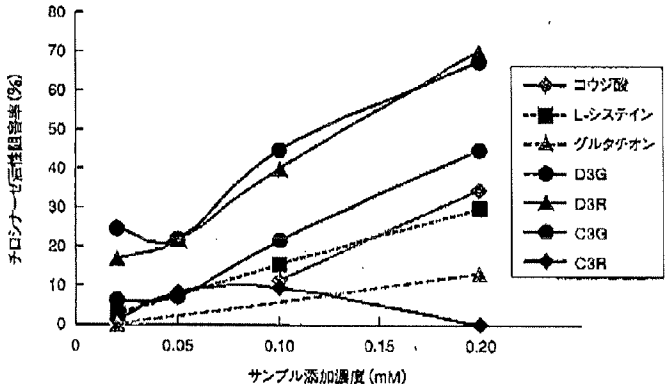
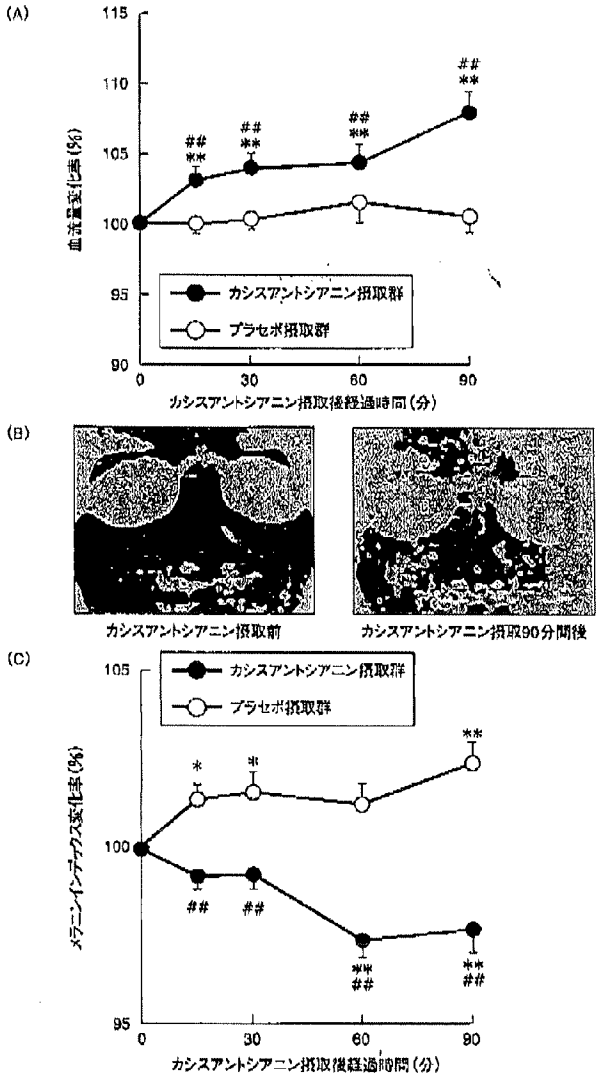



図1 カシスアントシアニンのチロシナーゼ活性阻害
（文献4より一部改変して引用）

チロシナーゼ，ドーパ混合液にD3G，D3R，C3G，C3R，対照としてコウジ酸，L-システイン，グルタチオンを0.025～0.2mM添加し，生成されるドーパクロム量を測定した。カシスアントシアニン無添加時のドーパクロムを100として，その生成量と比較することでチロシナーゼ活性阻害率を算出した。



Exhibit A
page 3




◎図2◎ カシスアントシアニン摂取による顔面血流とメラニンインデックスの変化

(文献5より一部改変して引用) ＊(B)は原図(カラー図)参照

(A) カシスアントシアニン摂取後の血流量変化率，(B) 典型的な顔面血流変化イメージ，(C) カシスアントシアニン摂取後のメラニンインデックス変化率

摂取前値との比較：* $p<0.05$，** $p<0.01$，プラセボ群との比較：## $p<0.01$，平均値±標準誤差



Exhibit A
page 4

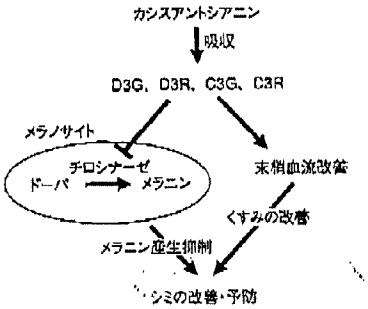



図3 カシスアントシアニンのシミ改善・予防効果仮説

ルコシド），C3R（デルフィニジン-3-ルチノシド）で構成されている。アントシアニンは主に胃部と小腸上部から吸収され末梢循環を改善することが知られている[5]。したがって，カシスアントシアニンを経口摂取することにより，末梢循環不良による肌色の赤みの低下などで生じるくすみに対して有効である可能性が考えられる。同時に，チロシナーゼ阻害活性が認められたため，カシスアントシアニンのシミに対する作用について検討した。

チロシナーゼを用い，D3G，D3R，C3G，C3Rを各々0.025～0.2mM添加した際のドーパから生成されるドーパクロム量をサンプル無添加時の生成量と比較評価した[6]。D3G，D3R，C3G，C3R添加時のチロシナーゼ活性阻害率は，0.2mM D3Gは67.5%，0.2mM D3Rは70.1%，0.2mM C3Gは45%とコウジ酸やLシステインより高いチロシナーゼ阻害活性を示した（図1）。

以上のことから，ヒトでシミを改善・予防する効果を検討した。30～45歳の健常女性被験者33名にカシスアントシアニン50mgを含む飲料100mLとカシスアントシアニンを含まない飲料100mLとを単回摂取するクロスオーバー二重盲検試験を行い，摂取後の顔の血流量変化をレーザードップラー血流計で，内眼角下部のメラニンインデックスをメグザメーターMX18で測定し，群間で比較した[6]。その結果，カシスアントシアニン摂取群はプラセボ群と比較して摂取15分後より顔の血流量が有意に増加し，メラニンインデックスが有意に低い値を示した。この結果から，カシスアントシアニン摂取により，皮膚色が薄くなることが示唆された（図2）。しかし，このヒト試験は単回摂取試験であるため，今後長期摂取による検証が望まれる。

以上の結果をまとめると，摂取したカシスアントシアニンが吸収され，メラノサイトに作用し，チロシナーゼ活性を阻害することと血流改善作用によりくすみを改善することにより，シミの改善・予防に働くことが期待できる（図3）

## 2 シワの改善と予防効果

シワは，生理的老化による乾燥と細胞の機能低下で起こるコラーゲン線維，弾性線維などの細胞外マトリクス量の低下によって組織が萎縮することで発生する。また，光老化においては紫外線に曝露されること

日立原町電子工業(株) ■ 微小循環モデル測定装置 ■ 1/1 ページ

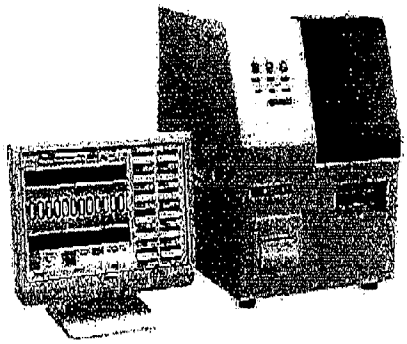
MCFAN Micro Channel Array Flow Analyzer

装置外観

赤血球変形能、白血球活性度が一目で観察できます。
血液の流れを観察できます！

MCFANは毛細血管を模擬し、簡単な操作で血液の流れを直接顕微鏡観察・記録が出来る装置です。予防医学や健康食品、製薬関連の研究開発用として、お役立て下さい。

エムシーファン
(HR300)



装置に関するお問合せは
株式会社エムシー研究所へ
http://www.mclab.co.jp/

NEXT>

ホームへ



このページの最初に戻る

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page 1

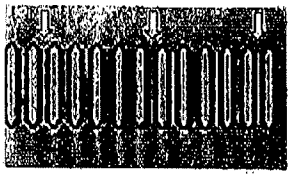
日立原町電子工業(株) ■ 微小循環モデル測定装置 ■ 1/1 ページ

MCFAN Micro Otuninal Array Flow Analyzer

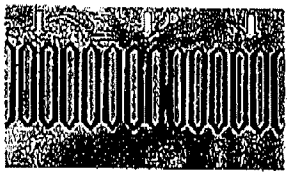
## 装置の特長

特長

1. 毛細血管を模擬したシリコンチップ流路にて、血液の流れを直接モニターで観察できます。



サラサラ状態



ドロドロ状態

※写真:日本レオロジー学会提供

2. 流路を血液が流れる通過時間を測定できます。
3. 流路を通過する細胞の変形状態をモニターで観察できます。

使用例

1. 赤血球変形能の観察
2. 白血球活性度(粘着性)の観察
3. 血小板凝集能の観察

シリコンチップ

1. 流路幅4μm～7μmを標準チップとし、目的に合わせて選択できます。
2. 流路形状をカスタムデザインする事により、装置の応用範囲が広がります。

<BACK NEXT>



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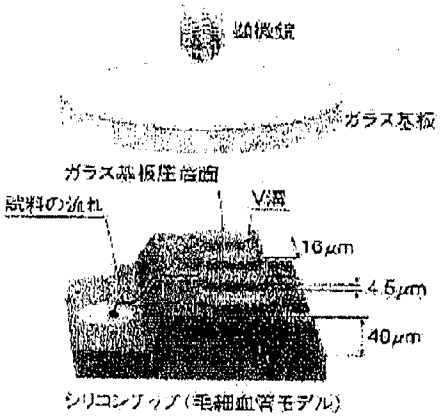
日立原町電子工業(株) ■ 微小循環モデル測定装置 ■ 1/1 ページ

MCFAN Micro Channel Array Flow Analyzer

測定原理

・観察部イメージ図




・試料の流れイメージ図

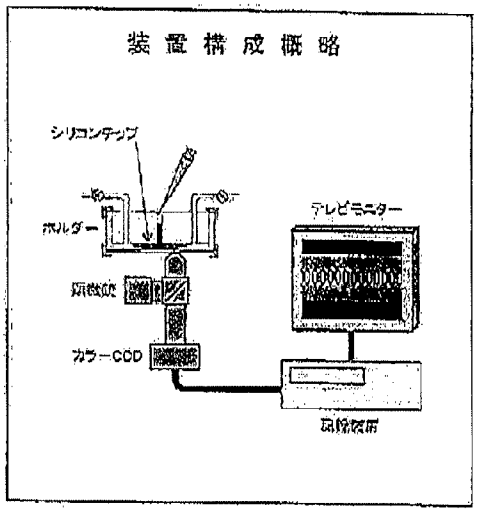



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EXHIBIT B
page 3

日立原町電子工業(株) ■ 微小循環モデル測定装置 ■ 1/1 ページ

MCFAN Micro Channel Array Flow Analyzer

測定事例

サラサラ状態　　ドロドロ状態

血液が毛細血管モデルをスムーズに流れている状態

血液が毛細血管モデルに凝集している状態

<BACK NEXT>



ホームへ
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Vascular Pharmacology
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# Possible mediators involved in decreasing peripheral vascular resistance with blackcurrant concentrate (BC) in hind-limb perfusion model of the rat

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**Abstract**

We analyzed mechanisms decreasing hind-limb perfusion pressure (PP) with blackcurrant concentrate (BC) in the rat. The decrease in PP with BC was abolished by endothelial removal, nitroarginine plus indomethacin, nitroarginine plus catalase or 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one as an inhibitor of guanylyl cyclase and potassium channel(s), and accompanied by the increased cyclic GMP level. Partial but significant inhibition owed by KCl was observed during combination. Authentic $H_2O_2$ decreased the PP in a sensitive manner to catalase and tetraethylammonium. The decrease in PP with BC in the presence of nitroarginine was significantly enhanced by diverse potassium channel blockers. Two cyclooxygenase metabolites of 4 endocyanins purified from BC definitely decreased the PP through similar mechanisms to BC. These results suggest that the decreased PP with BC is possibly mediated by endothelial NO and $H_2O_2$, and partially through the activation of diverse potassium channels, and that 2 cyclooxygenase metabolites play a major role as active components of BC.


*Keywords:* Decrease in peripheral vascular resistance; Delphinidin; Endothelium-derived hyperpolarizing factor; $H_2O_2$; NO

## 1. Introduction

The blackcurrant (*Ribes nigrum* L.) concentrate (BC), which had been prepared from blackcurrant juice, is reportedly known to increase resting forearm blood flow (Matsumoto et al., 2005) and to improve the peripheral circulation disorder caused by the cold water loading in human volunteers (Takenami et al., 2004). Furthermore, BC intake effectively restored the decreased oxy-hemoglobin level during and after the operating workload, and the root mean square value of electromyograph leaded to increase with BC, leading to an improvement of muscle fatigue (Takenami et al., 2003). Although BC relaxes the aortic strips of the rat through increasing endothelium-dependent NO production (Nakamura et al., 2002), the mechanisms improving the peripheral circulation with BC remain to be clarified.

Peripheral vascular resistance is controlled by the vascular tone of peripheral resistance vessels, in which endothelial cells play an important role. NO has been characterized as the endothelium-derived relaxing factor (Palmer et al., 1987; Ignarro, 1990) and as a main substance controlling the vascular tone (Moncada et al., 1988). Meanwhile, NO- and prostacyclin-independent, endothelium-dependent relaxation associated with hyperpolarization has been increasingly recognized to be important in the maintenance of vascular tone mainly in the peripheral resistance vessels, giving rise to be still unidentified endothelium-derived hyperpolarizing factor (EDHF) (Fuchsgott and Vanhoutte, 1989; Triggle and Ding, 2002). According to Ndiaye et al. (2003), NO and EDHF were involved in the relaxation produced by red wine polyphenols in the porcine coronary arteries. Parkington et al. (2002)

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demonstrated that EDHF seems to be more important in decreasing the vascular tone with acetylcholine in a hind-limb perfusion experiment using the rat. Thus, we designed the present experiments to investigate whether BC decreases the peripheral vascular resistance in the hind-limb perfusion model of the rat, if so, to analyze the mechanisms. Since BC contains 4 anthocyanins (Matsumoto et al., 2001), we also investigated the involvement of these anthocyanins in generating the biological activity of BC.

## 2. Materials and methods

### 2.1. Experimental animals

This study complied with the Animal Welfare Regulations of Tokyo Medical and Dental University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society. 13- to 15-week-old Sprague-Dawley rats were purchased and kept in the Animal Care Center of our university for 1 week before the experiments.

### 2.2. Chemicals

A powdered BC and 4 anthocyanins, delphinidin-3-rutinoside (D3R), delphinidin-3-glucoside (D3G), cyanidin-3-rutinoside (C3R), and cyanidin-3-glucoside (C3G) were prepared from a commercially available blackcurrant juice according to the method described previously (Matsumoto et al., 2001). The purity of 4 anthocyanins was more than 99.5%, and the chemical structure of each compound was confirmed by UV, FAB-MS, and NMR spectra analyses (Matsumoto et al., 2001). Major components of BC were determined to be 10.83% anthocyanins consisting of 5.09% D3R, 1.48% D3G, 3.26% C3R, and 0.50% C3G (Matsumoto et al., 2001).

Carbenylcholine chloride (carbachol), $N^G$-nitro-L-arginine (nitroarginine), indomethacin, charybdotoxin, apamin, iberiotoxin, ouabain, 3-isobutyl-1-methylxanthine (IBMX), 4-aminopyridine, glibenclamide, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ), catalase, 17-octadecynoic acid (17-ODA), carbenoxolone, arachidonylethanolamide (anandamide) and hemin were purchased from Sigma (St. Louis, MO, USA). Phenylephrine hydrochloride (phenylephrine) was from Kowa Company (Nagoya, Japan). Triton X-100, ethylenediaminetetraacetic acid (EDTA) and tetraethylammonium were from Nacalai Tesque (Kyoto, Japan), zinc protoporphyrin was from Porphyrin Products (Utah, USA), and KCl, $H_2C_2$ and $BaCl_2$ were from Wako Pure Chemicals (Osaka, Japan). BC, phenylephrine, carbachol, nitroarginine, charybdotoxin, apamin, iberiotoxin, carbenoxolone, $BaCl_2$, glibenclamide, catalase, tetraethylammonium, KCl, ouabain, $H_2O_2$, EDTA and Triton X-100 were dissolved in distilled water except for indomethacin, IBMX, and 17-ODA in dimethylsulfoxide, anandamide in ethanol, and hemin and zinc protoporphyrin in pyridine (Lyall et al., 2000). BC, IBMX and catalase were dissolved immediately before use. Other agents were kept as stock solutions until use and were periodically replaced. Catalase solution was used after the filtration.

### 2.3. Perfusion experiments

The animals were heparinized (200 IU/kg, i.p.) to avoid coagulation and then sacrificed by dislocation and exsanguination from carotid arteries under ether anesthesia. A 21-gauge metal catheter was inserted into the proximal portion of the femoral artery, the proximal portion of the femoral vein was opened longitudinally to facilitate the flow, and the hind-limbs were separated from the rest of the body. Modified Krebs solution was infused into femoral artery with the aid of peristaltic pump at a constant flow rate of 3.5 ml/min. The perfusion solution was prepared by mixing 2 volumes of Krebs solution with 1 volume of Dulbecco's Modified Eagle's Medium (DMEM). The rate of mixing between Krebs solution and DMEM was determined to minimize the occurrence of edema in the preliminary experiments. The perfusion of a cell- and protein-free solution into an isolated limb was accompanied by significant edema, which was probably associated with endothelial dysfunction. This latter condition was a significant concern in our model. Thus, some manipulations of the perfusion solution and temperature conditions were performed in order to achieve no edema and reproducibility of the experimental results according to the previous report (Loyaga et al., 2002; Loyaga-Rendon et al., 2005). The magnitude of edema was definitely less at low temperature and by using modified Krebs solution. In a preliminary experiment, we confirmed that the responsiveness of hind-limb vascular bed to phenylephrine and carbachol did not differ between 22 °C and 37 °C, thus all experiments were performed at 22 °C (Loyaga et al., 2002; Loyaga-Rendon et al., 2005). The changes in perfusion pressure were continuously monitored and recorded on a pen-writing oscillograph for analyses.

### 2.4. The decreases in the perfusion pressure with BC, anthocyanins, $H_2O_2$, anandamide, hemin or low concentration of KCl

20 min after starting the perfusion, a stable basal perfusion pressure was attained. In order to confirm the presence of functioning endothelium, carbachol in a concentration of 100 μM was infused during the contraction caused by 10 μM phenylephrine. 30 min after washout of carbachol and phenylephrine, the effects of bolus application of BC in concentrations of 1.2 to 2.5 μg/ml, 10 and 100 μM $H_2O_2$, 10 μM anandamide, 3.3 μM hemin, and 2 to 11 mM KCl were determined. Since 1.8 μg/ml BC contains 150 nM D3R, 50 nM D3G, 100 nM C3R, 18 nM C3G, the effects of 4 anthocyanins alone at these concentrations or the reconstructed mixture of these anthocyanins were also determined. Results were compared with those obtained as the paired-time controls in the contra-lateral hind-limb without application of test agent.

### 2.5. Pharmacological analyses of the decrease in perfusion pressure with BC

Nitroarginine (30 μM) as an inhibitor of NOS, tetraethylammonium (1 mM) as a non-selective blocker of potassium channels, indomethacin (1 μM) as a cyclooxygenase inhibitor, and ODQ (10 μM) as an inhibitor of guanylyl cyclase and potassium channel(s) were used for pharmacological analyses of the decrease in perfusion pressure with 1.8 μg/ml BC. The BC-induced decrease in the perfusion pressure in the presence of nitroarginine (30 μM) was analyzed using catalase (1250 U/ml), carbenoxolone (30 and 100 μM) as an inhibitor of gap junctions, 17-ODA (3 μM) as an inhibitor of cytochrome *P*-450 monooxygenase and zinc protoporphyrin (3.3 μM) as an inhibitor of heme oxygenase-1. These agents except for catalase were applied 20 min prior to the phenylephrine contraction. Catalase was added 30 min prior to the phenylephrine contraction. The contra-lateral hind-limb in the presence or absence of nitroarginine was used as a paired-time control. Triton X-100 (0.05%) applied for 1 min was used to examine the endothelium dependence of the decrease in perfusion pressure with BC. In order to cause the contraction with 60 mM KCl, 60 mM NaCl in the modified Krebs solution was replaced by 60 mM KCl.

In the presence of 30 μM nitroarginine and 1 μM indomethacin, the involvement of potassium channels in decreasing the perfusion pressure with BC (1.8 μg/ml) was investigated. For this purpose, several blockers of potassium channels such as tetraethylammonium (1 mM), charybdotoxin (100 nM), apamin (100 nM), iberiotoxin (100 nM), glibenclamide (3 μM), 4-aminopyridine (1 mM) and $BaCl_2$ (30 μM) were used. Ouabain (100 μM) was used as an inhibitor of Na/K ATPase. These agents were applied 5 min prior to the phenylephrine contraction. The contra-lateral hind-limb was used as a paired-time control in the presence of nitroarginine and indomethacin.

### 2.6. Measurement of cyclic GMP

In the preliminary study using rings of rat thoracic aorta, we found that the cyclic GMP levels in response to carbachol were increased both in the aortic tissue and the incubation medium (Krebs solution). There was a positive and significant ($P=0.001$) correlation ($r=0.806$) between cyclic GMP levels in the aortic tissue and incubation medium. Thus, we assumed that the detected cyclic GMP levels in the perfusate of the hind-limb would reflect changes in the tissue cyclic GMP (Loyaga-Rendon et al., 2005).

The isolated hind-limbs were perfused with modified Krebs solution containing 10 μM IBMX as a nonselective inhibitor of phosphodiesterases. The perfusate was collected for 10 min through the cannula inserted into the proximal femoral vein, and immediately frozen in liquid nitrogen and stored at −80 °C until analyses. Samples were collected before and during the stimulation with 1.8 or 2.5 μg/ml BC in the presence or absence of 30 μM nitroarginine and 1 mM tetraethylammonium. The stored samples were thawed and EDTA was added (at a final concentration of 20 mM), then the perfusate was concentrated by 1:20 vol. using a centrifugal vaporizer (CVE-100D, Eyela, Tokyo, Japan). The levels of cyclic GMP in the concentrate were assessed using radioimmunoassay kit according to the industry's instruction (Yamasa Shoyu, Tokyo, Japan).



### 2.7. Statistics

Results are given as mean ± S.E.M. of the number of experiments indicated. The extent of decrease in the perfusion pressure was expressed as percentage decrease to the contraction induced by phenylephrine or KCl. The $E_{max}$ (maximum decrease in the perfusion pressure) and $EC_{50}$ (concentration producing 50% of the $E_{max}$) values were obtained from the concentration–response curves. Results were compared using paired and unpaired *t*-test. $P<0.05$ was regarded as significant difference.

## 3. Results

### 3.1. Decrease in the perfusion pressure with BC

The basal hind-limb perfusion pressure, which had been measured 30 min after the test response with phenylephrine and carbachol, was determined to be 37.7±0.7 mmHg ($n=34$). Phenylephrine (10 μM) caused a sustained and stable contraction, of which extent was 141.7±3.9 mmHg ($n=34$). BC produced a sustained and progressive decrease in the perfusion pressure during the contraction caused by phenylephrine (Fig. 1A). After adding BC, the pressure gradually decreased and became the maximum approximately 30 to 40 min later. BC (1.2–2.5 μg/ml) produced a concentration-dependent decrease in the perfusion pressure with the maximum value of 77.0±6.4% at a concentration of 2.1 μg/

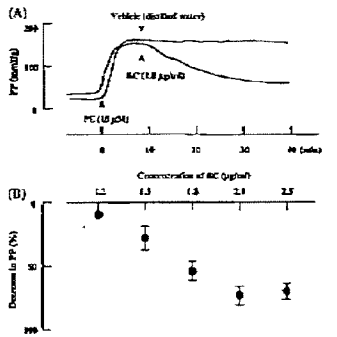


Fig. 1. Decrease in the perfusion pressure (PP) with blackcurrant concentrate (BC) in the hind-limb perfusion model of the rat. A bolus application of BC to a concentration of 1.8 μg/ml produced a sustained and progressive decrease in the perfusion pressure during the contraction caused by 10 μM phenylephrine (PE) as shown in (A). BC (1.2–2.5 μg/ml) produced a concentration-dependent decrease in the perfusion pressure with the maximal value of 77.0±6.4% at a concentration of 2.1 μg/ml (B). Each point represents the mean value of 5 to 10 independent determinations.

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Fig. 6. Effect of typical potassium channel blockers on the decrease in perfusion pressure with BC. All experiments were performed in the presence of 30 pM nitrergoline (LNA) and 1 mM tetraethylammonium (TEA). One mM 4-aminopyridine, 100 mM barium chloride, 100 nM iberiotoxin, 100 nM charybdotoxin, 150 nM apamin and 3 pM glibenclamide were used as analogs. * and ** significant differences at $P<0.05$ and $P<0.005$ vs. the pertinent control, respectively ($n=4$) except for $BaCl_2$ ($n=5$) and glibenclamide ($n=6$).

and 97.6±4.0% of the control for apamin ($n=4$)). In addition, the decrease in perfusion pressure with BC in the presence of 30 μM nitrergoline and 1 μM indomethacin remained unaffected by 3 μM glibenclamide as a blocker of ATP-sensitive potassium channel ($K_{ATP}$) (Jackson et al., 1997) (89.0±19.9% of the control, $n=5$).

### 3.6. Evaluation of major components of BC to decrease the perfusion pressure

Based on the percentage contents of the major components in BC, the concentrations of D3R, D3G, C3R and C3G, which are contained in 1.8 μg/ml BC, were calculated to be 150 nM, 50 nM, 100 nM, and 18 nM, respectively. We evaluated whether these anthocyanins decrease the perfusion pressure. The extent of the decrease in perfusion pressure was determined to be 23.4±0.48% with 150 nM D3R ($n=4$), 15.0±0.48% with 50 nM D3G ($n=4$), 5.9±1.4% with 100 nM C3R ($n=4$), and 5.4±1.2% with 18 nM C3G ($n=4$). These results are shown in Table 1. The reconstructed mixture with these 4 anthocyanins produced a definite decrease in the perfusion pressure (44.4±6.2%, $n=5$), of which value was not significantly different from that obtained for 1.8 μg/ml BC (35.3±6.9%, $n=9$). D3R and D3G of 4 anthocyanins produced a definite, sustained and progressive decrease in the perfusion pressure. In addition, the extent of decrease in perfusion pressure with C3R and C3G was only slight even at approximately 10 times higher concentrations (5.3±2.2% for 1 μM C3R, $n=4$) and (3.6±0.8% for 150 μM C3G, $n=4$). Among the anthocyanins contained in BC, we selected D3R and D3G as major components for the pharmacological analyses. 30 μM nitrergoline alone partially but significantly attenuated the decrease in perfusion pressure with 150 nM D3R (62.4±1.9% of the control, $n=4$, $P<0.005$) or 50 nM D3G (71.9±9.1% of the control, $n=4$, $P<0.05$) as shown in Fig. 7A and B. Nitrergoline (30 μM) in combination with tetraethylammonium (1 mM) greatly attenuated the decrease in perfusion pressure with 150 nM D3R (20.6±5.4% of the control, $n=4$, $P<0.005$) or 50 nM D3G (26.3±8.2% of the

Table 1
Decrease in perfusion pressure (PP) with 4 major anthocyanins contained in the black currant fruit concentrate (BCC) (A) and their key effects of nitrergoline (LNA) in combination with tetraethylammonium (TEA) (B) or carotene (C) on the decreased PP with D3R and D3G

(A)

| | D3R | D3G | C3R | C3G |
|---|---|---|---|---|
| Decrease in PP (%) | 23.4±0.4 | 15.0±0.5 | 5.3±1.4 | 5.4±1.2 |

(B)

| | D3R | D3G |
|---|---|---|
| Control | 25.3±1.0 | 15.8±1.0 |
| LNA + TEA | 5.2±1.4*** | 4.2±1.5*** |
| [% of control] | (20.6±5.5) | (26.3±8.2) |

(C)

| | D3R | D3G |
|---|---|---|
| Control (+LNA) | 15.8±0.6 | 11.4±1.1 |
| LNA + Carotene (% of control) | 8.7±0.4*** (51.8±5.7) | 5.7±1.0*** (52.3±9.2) |

Based on the percentage contents of 4 anthocyanins in 1.8 μg/ml BC, the concentrations of D3R, D3G, C3R, and C3G were calculated to be 150 nM, 50 nM, 100 nM and 18 nM, respectively. D3R and D3G of 4 anthocyanins at concentrations tested definitely decreased the perfusion pressure as shown in (A) ($n=4$). The decrease in perfusion pressure with D3R and D3G were significantly ($P<0.005$) inhibited by 30 μM nitrergoline (LNA) in combination with 1 mM tetraethylammonium (TEA) as shown in (B) ($n=4$). and 30 μM nitrergoline (LNA) in combination with 1250 U/ml catalase as shown in (C). 30 μM nitrergoline (LNA) alone partially but significantly ($P<0.005$ and $P<0.05$) attenuated the decrease in perfusion pressure with D3R and D3G as shown in the results in (B) and (C).




Fig. 7. Pharmacological analyses of the decrease in perfusion pressure with D3R (A) and D3G (B) in Ni-drip perfusion model of the rat. The decrease in the perfusion pressure with D3R (A) or D3G (B) was significantly attenuated by 30 μM nitrergoline (LNA) alone, 1 mM tetraethylammonium (TEA) or 1250 U/ml catalase in the presence of 30 μM LNA. * and ***Significant difference at $P<0.05$ and $P<0.005$ vs. paired time-matched control, respectively ($n=4$).

control, $n=4$, $P<0.005$) as shown in Fig. 7A and B. Catalase at a concentration of 1250 U/ml also greatly attenuated the decrease in perfusion pressure with D3R (14.6±2.4% of the control, $n=4$, $P<0.005$) or D3G (23.8±7.4% of the control, $n=4$, $P<0.005$) in the presence of 30 μM nitrergoline as shown in Fig. 7A and B.

## 4. Discussion

It has been reported that oral intake of BC significantly improves the peripheral circulation in human volunteers (Takawaki et al., 2003, 2004; Matsumoto et al., 2005). However, mechanisms improving the peripheral circulation remain to be clarified.

BC caused a sustained and progressive decrease in the hind-limb perfusion pressure, which was abolished by endothelial denudation, nitrergoline as an inhibitor of NOS in combination with tetraethylammonium as a non-selective blocker of potassium channels or by ODQ used as inhibitors of guanylyl cyclase (Moro et al., 1996) and potassium channels (Adeagbo, 1999), and partially but significantly attenuated by nitrergoline or tetraethylammonium alone and under the depolarization. Furthermore, the decrease in perfusion pressure with BC in the presence of tetraethylammonium was accompanied by the increased cyclic GMP production, which was abolished by nitrergoline. However, indomethacin failed to modify the change in perfusion pressure with BC. These results suggest that the decrease in perfusion pressure with BC is endothelium-dependent and possibly mediated by NO and EDHF, but not by vasodilator prostanoids(s). It is reportedly known that ODQ acts also as an inhibitor of the potassium channel(s) (Bolduc et al., 2001) in addition to the inhibition of guanylyl cyclase (Moro et al., 1996). Furthermore, we observed that the decrease in perfusion pressure with 1-ethyl-2-benzimidazolinone (1-EBIO) (1–100 μM), which is known as an activator of $K_{Ca}$ channel (Devor et al., 1996), was inhibited by ODQ (Loyaga-Rendon et al., 2005). Taken together, they further support the speculation that the decrease in perfusion pressure with BC is mediated by not only NO but also EDHF.

We tried to analyze the possible mediators in decreasing the perfusion pressure with BC. In the presence of nitrergoline, catalase as an endogenous peroxidase abolished the decrease in perfusion pressure with BC. Meanwhile, the inhibitory effect of catalase disappeared, after heating the enzyme solution at 60 °C for 30 min. In addition, exogenously applied $H_2O_2$ produced a sustained and progressive decrease in the perfusion pressure in a similar manner to that BC did. Furthermore, the decrease in perfusion pressure with $H_2O_2$ was abolished by catalase and tetraethylammonium. These results suggest that the decrease in the perfusion pressure with BC is partially mediated by $H_2O_2$.

Endogenous $H_2O_2$ has been known to be a mediator to relax the mesenteric arteries of humans and rodents (Matoba et al., 2000; Hamilton et al., 2001). $H_2O_2$-induced relaxation in canine middle cerebral arteries was significantly attenuated by $BaCl_2$ as a $K_{ir}$ channel blocker or 4-aminopyridine as a $K_V$ channel blocker (Ida and Kanaide, 2000). Furthermore, the relaxation caused by $H_2O_2$ was inhibited by iberiotoxin as a $BK_{Ca}$ channel blocker in the rat superior mesenteric arteries (Gao et al., 2004) and by the combination of charybdotoxin as a $BK_{Ca}$/$IK_{Ca}$ channel blocker with apamin as an $SK_{Ca}$ channel blocker in human coronary arterioles (Miura et al., 2003). However, glibenclamide as a $K_{ATP}$ channel blocker failed to modify the relaxation induced by $H_2O_2$ in human coronary arterioles (Miura et al., 2000). If these findings are considered together, they suggest that $H_2O_2$ produces the relaxation through activation of diverse potassium channels in the different kind of vascular tissues. In the present experiments, we demonstrated that the decrease in perfusion pressure with BC in the presence of nitrergoline was significantly attenuated by tetraethylammonium, $BaCl_2$, 4-aminopyridine, iberiotoxin and charybdotoxin in combination with apamin, but remained unaffected by glibenclamide. These results suggest that diverse potassium channels possibly activated by endogenous $H_2O_2$, which would be generated by BC, are involved in decreasing the perfusion pressure with BC. Although charybdotoxin and apamin alone failed to modify the decrease in perfusion pressure with BC in the present experiments, Matoba et al. (2000) and Miura et al. (2003) have demonstrated that the

relaxation caused by $H_2O_2$ was inhibited only by the combination of charybdotoxin with apamin in mouse mesenteric arteries and human coronary arterioles. However, since the $H_2O_2$-induced relaxations in rat superior mesenteric arteries (Gao et al., 2004) and canine middle cerebral arteries (Iida and Katusic, 2000) were attenuated by charybdotoxin alone, further experiments should be performed to clarify the discrepancy between our data and the previous reports. On the other hand, no reports describing the effect of apamin alone on the $H_2O_2$-induced relaxation have been found.

In addition to $H_2O_2$, several candidates for EDHF such as anandamide as an endocannabinoid (Randall and Kendall, 1997; Giuffrida et al., 2001), carbon monoxide (Villanoor et al., 2000), epoxyeicosatrienoic acids (EETs) (Campbell et al., 1996; Zhang et al., 2001; Fleming, 2004), and potassium ion (Dora and Garland, 2001; Edwards et al., 1998; Neild et al., 2003) have been proposed. The gap junction has also been proposed to play a pivotal role for relaxing vascular smooth muscle (Hutcheson and Griffith, 2000; Griffith et al., 2004). In the present experiments, we examined whether these mediators were implicated in decreasing the perfusion pressure with BC. The decrease in perfusion pressure with BC in the presence of nitroarginine remained unaffected by 17-ODA as an inhibitor of cytochrome *P*-450 monooxygenase, zinc protoporphyrin as an inhibitor of heme oxygenase-1 or carbenoxolone as an inhibitor of gap junction, suggesting that metabolite(s) of cytochrome *P*-450 monooxygenase, carbon monoxide or the gap junction is not involved in decreasing the perfusion pressure with BC. Furthermore, the decrease in perfusion pressure with BC may not be mediated by endocannabinoid, carbon monoxide or 11,12-EET as one of the metabolites of cytochrome *P*-450 monooxygenase, since authentic anandamide, hemin as a substrate of heme oxygenase-1 and authentic 11,12-EET (Loyaga-Rendon et al., 2005) failed to modify the perfusion pressure. We also examined the possible involvement of potassium ion. Low concentration of KCl (2–11 mM) (note that modified Krebs solution contains 4.8 mM basal KCl) produced a definite decrease in the perfusion pressure (Loyaga-Rendon et al., 2005), which was effectively inhibited by ouabain as a Na/K ATPase inhibitor, but remained unaffected after the endothelial removal (Loyaga-Rendon et al., 2005). While, the decrease in perfusion pressure with BC was resistant to the former, but sensitive to the latter, ruling out the possible involvement of potassium ion.

Finally, we tried to examine the ability of major components contained in BC to decrease the perfusion pressure. Only D3R and D3G of 4 anthocyanins at concentrations contained in 1.8 μg/ml BC produced a definite, sustained and progressive decrease in the perfusion pressure in a similar manner to BC. Since the potency of C3R and C3G was only slight even at approximately 10 times higher concentrations than calculated ones, we assumed that D3R and D3G are major components of BC. The extent of decreases in the perfusion pressure with D3R and D3G in the presence of nitroarginine was greatly attenuated by tetraethylammonium or catalase as in the case of BC, and partially inhibited by nitroarginine, leading us to assume that D3R and D3G decrease the perfusion pressure through the similar mechanisms to BC.

## 5. Conclusions

We concluded that the decrease in perfusion pressure with BC is possibly mediated by endothelial NO and $H_2O_2$, and partially through activation of diverse potassium channels. Furthermore, D3R and D3G of 4 anthocyanins play an important role as major components of BC.

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第3回(平成16年度) IBB BioFuture Research Encouragement Prize 研究発表会要旨
制御分野　博士課程の部)

# カシス（*Ribes nigrum* L.）抽出物による末梢血管抵抗低下機序

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【目的】「カシスポリフェノール(以下BCと記す)」は、カシス濃縮果汁を粉末状にした機能性食品素材である[1]。BCのヒトでの視覚機能改善効果[2]ならびに末梢血流改善効果[3, 4, 5]が確認されているが、作用機序は不明である。BCにはdelphinidin-3-glucoside (D3G)、delphinidin-3-rutinoside (D3R)、cyanidin-3-glucoside (C3G)、およびcyanidin-3-rutinoside (C3R)の4種のアントシアニンが含まれているので、BCのラット後肢末梢抵抗血管拡張作用機序を詳細に解析するとともに、4種のアントシアニンの寄与についても併せ検討した。
【方法と結果】ラット内腸骨動脈内に挿入したカニューレを介して改変クレブス液を定流量還流し、還流圧の変化を記録した。Phenylephrine ($10^{-6}$M) 誘発収縮下にBCを添加すると還流圧は徐々に低下した(Fig.1)。同作用は濃度依存的で(Fig.2)、cyclic GMP産生増加を伴っており、血管内皮除去後には消失した。さらに同作用は、一酸化窒素合成酵素阻害剤(nitroarginine)と非特異的$K^+$チャネル阻害剤(tetraethylammonium)との併用またはnitroarginineとcatalaseとの併用により完全に阻害された。$H_2O_2$標品によってBC作用に類似の還流圧低下が観察され、catalaseならびにtetraethylammoniumはこれを抑制した。また、nitroarginine存在下、BC誘発還流圧低下は、薬理学的性質の異なる特異的$K^+$チャネル阻害剤(barium chloride, iberiotoxin, 4-aminopyridine, charybdotoxin + apamin)によって部分的に抑制された。作用強度は異なるものの4種のアントシアニンは何れも還流圧低下作用を示し、アントシアニン作用の総和はBC作用に匹敵した。また、主要アントシアニン、D3GならびにD3R作用はBCの場合と同様、nitroarginine + tetraethylammonium またはnitroarginine + catalaseにより阻止された。
【結論】BCの末梢血管抵抗低下作用は血管内皮依存性でありNOおよび過分極因子(EDHF)産生／遊離の増加を介して惹起されることが示唆された。さらに、$H_2O_2$がEDHFの有力候補物質と考えられ、種類の異なる複数の$K^+$チャネルを活性化する結果、過分極と末梢血管拡張をもたらす可能性が示唆された。BCの末梢血管抵抗低下作用において4種のアントシアニンが主要な役割を果たしている可能性が示唆された。

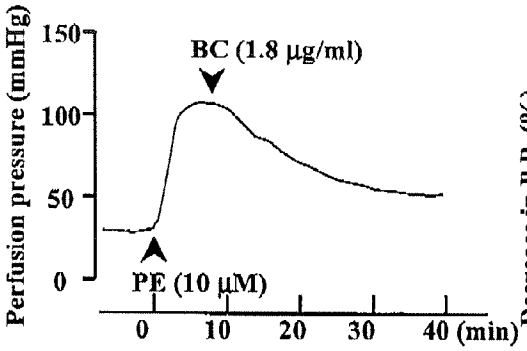


**Figure 1.** *A sustained and progressive decrease in the perfusion pressure produced by blackcurrant concentrate (BC) in a concentration of 1.8ug/ml during the contraction caused by 10μM phenylephrine (PE).*

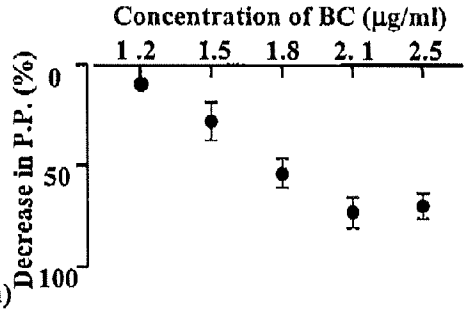


**Figure 2.** *Concentration-dependent decrease in the perfusion pressure with blackcurrant concentrate (BC) in the hind limb perfusion model of the rat.*

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Exhibit C
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